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Genetic Variation and Association Analysis of Phenolic Compounds in Rapeseed (*Brassica napus* L.) Mutant Lines Using Genotyping-by-Sequencing (GBS)

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Abstract: The concentration of phenolic compounds in rapeseed is important because they are either anti-nutritional compounds or directly related to antioxidant activity. In this study, single nucleotide polymorphisms (SNPs) were identified using genotyping-by-sequencing (GBS), and an association study was conducted to investigate phenolic content in 95 rapeseed mutant lines derived from gamma rays and their original cultivar. A total of 3,196,318 SNPs were detected, resulting in the identification of a set of 70,208 union SNPs used to perform association studies. Seven compounds were identified, sinapine being the major phenolic compound (91.2–99.2%) in all genotypes. An association study was conducted for seven compounds and total phenolic content (TPC). It identified 241 SNPs that were significantly associated with these compounds, total sinapine content (TSC), and TPC. Based on the SNP markers detected, *BnaC02g20420D* was associated with dihexose, *BnaC08g30570D* with progoitrin, *BnaA01g06890D* with methyl sinapate, *BnaA09g16810D* with sinapine(4-*O*-8′)guaiacyl, *BnaCnng39930D* with *trans*-sinapine 1, *BnaA07g31720D* with *trans*-sinapine 2, *BnaC03g31950D* with sinapoyl malate, and *BnaAnng27700D* with TPC. These were selected as candidate genes that may play a key role in rapeseed. The SNP markers associated with these key phenolic compounds can be used as targets in breeding programs to reduce anti-nutritional components.

Keywords: rapeseed; mutation breeding; phenolics; SNP; GBS; association study

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1. Introduction

Rapeseed (*Brassica napus* L., Brassicaceae) is one of the most widely cultivated crops. It produces beautiful flowers and is extensively used as food, animal feed, and a resource for bio-industries [1,2]. Rapeseed seeds are used as both food and feed. They contain high levels of proteins, minerals, carotenoids, and vitamins and have antioxidant properties. The presence of high amounts of antinutritional compounds, such as phenolics, limits the use of rapeseed extracts in animal feed [3,4]. Breeding to reduce the concentrations of phenolic compounds is important for developing high-quality rapeseed for food and feed [4,5]. Similarly, genetic enhancement of seed yield is one of the main goals of rapeseed breeding

programs [1]. As one of the most important crops for the food and animal feed industries in Korea, rapeseed needs to be improved in terms of its nutritional value and seed yield, as the amount of rapeseed produced at present is insufficient to meet the national demand. Therefore, it is important to produce materials with novel nutritional traits and high seed yield to be used as rapeseed genetic resources.

Sinapate esters with sinapoylcholine (sinapine), a major phenolic compound in rapeseed seed, are known for their antioxidant properties and their role as important natural cancer-preventive agents [6,7]. However, the presence of phenolic compounds and glucosinolates in rapeseed confers a bitter taste and decreased palatability, thereby making food and feed less desirable. In addition, giving rapeseed meal containing sinapine to laying chickens can produce fishy-smelling eggs [5,6]. The phenylpropanoid pathway is the main metabolic pathway that produces phenolic compounds, with one of its major products being sinapine, accounting for up to 80% of the phenolic compounds in rapeseed seeds. Glucosinolates are nitrogen- and sulfur-containing compounds found in members of the Brassicaceae family, including rapeseed. Glucosinolate biosynthesis relies on interactions among several biosynthetic pathways in plants [8]. Plants contain more than 100 different glucosinolate compounds that can be chemically categorized into aliphatic, indolic, and aromatic glucosinolates [8,9]. Depending on their precursor amino acids, glucosinolates can be further categorized into indole glucosinolates derived from tryptophan; aliphatic glucosinolates derived from alanine, leucine, valine, and methionine; and aromatic glucosinolates derived from phenylalanine and tyrosine. Progoitrin (2R-2-hydroxy 3-butenyl glucosinolates) is an example of aliphatic glucosinolate, which serves as a precursor to sulforaphane [8–11]. Several studies have explored the connections among rapeseed phenolic compound biosynthesis, glucosinolate biosynthesis, and metabolic pathways [4,8]. Although glucosinolates and phenylpropanoids are synthesized through distinct biosynthetic pathways and have unique functions, a study on *Arabidopsis* mutants revealed interactions between glucosinolate biosynthesis and phenylpropanoid biosynthesis pathways [11]. In addition, glucosinolates are of significant interest due to their health benefits because of their anticancer effects [12]. However, these compounds confer a bitter taste and include biologically active isothiocyanates that reduce the quality of food and feedstocks made from rapeseed [1,3]. Since natural variation in sinapine and glucosinolate contents is limited in rapeseed, it is a reasonable approach to improve its nutritional value with mutations targeting sinapine content [6,7,13]. Radiation-induced mutation is one of the most effective methods for altering the composition of functional compounds for commercialization. Mutants obtained using radiation breeding methods often exhibit altered phenotypic traits, including changes in secondary metabolite profiles [14]. Gamma-ray radiation represents ionizing radiation that induces diverse mutational changes, from simple base substitutions to single and/or double-strand DNA breaks. In other studies, mutation breeding technology has been successfully used to improve the phenolic compound contents in various crops to levels surpassing those of natural variation [14–16]. While the phenolic and glucosinolate biosynthetic pathways have been extensively studied in rapeseed, the molecular networks that regulate phenolic and glucosinolate compound metabolism in mutant genotypes obtained using radiation breeding techniques are not well understood.

Entire plant genomes can be determined using next-generation sequencing (NGS), and the data can be used to detect single-nucleotide polymorphisms (SNPs) [17]. Genotyping-by-sequencing (GBS) evaluates the target genome using scaled-down labels. These methods allow for direct analyses and advancement of breeding goals using high-throughput SNP genotyping analysis, quantitative trait locus (QTL) mapping, and association mapping [18,19]. Association mapping is when SNPs are used for the indirect selection of target traits in crops [20–22]. There are non-random associations between SNP markers and nutritional characteristics in the mutants of genetically diverse crops. Association mapping has been widely applied in *Brassica* breeding to target nutritional characteristics such as protein, phenolics, and oil contents, fatty acid composition, and glucosinolates [5,7,13,14,22].

In this study, we developed novel rapeseed mutant lines from domestic rapeseed cultivars using gamma-ray irradiation and determined the phenolic and glucosinolate contents in these lines. The objective was to identify SNPs in rapeseed mutant lines derived from the original cultivars. Simultaneously, an association study using GBS analysis revealed associations between novel SNP loci and phenolic compound/glucosinolate contents in the mutant lines.

2. Materials and Methods

2.1. Plant Materials

The mutant lines were generated by treating the seeds of the commercial cultivar *Brassica napus* 'Youngsan' and *B. napus* 'Tammi' with various doses of gamma-ray (^{60}Co) irradiation as follows: 500 Gy (46 mutant lines from Tammi), 600 Gy (37 mutant lines; Y6 lines), 800 Gy (7 mutant lines; Y8 lines), and 1000 Gy (5 mutant lines; Y10 lines) at 2009. The treated seeds were sown to obtain the M_1 generation, and seeds from one silique developed from the main stem of each M_1 plant were harvested. In the M_2 generation, all individuals from the original cultivar were investigated for agronomic characteristics (seed yield, flowering time, and dwarfism) mutations. Ninety-five rapeseed mutants, selected based on their phenolic compound contents, were obtained from the M_7 and M_9 generations. The self-fertilization procedure was continued until the M_9 generation. We analyzed the uniformity of the phenolic contents using UPLC (Ultra Performance Liquid Chromatography) for three generations (M_7 to M_9) to select stable lines. Finally, 95 mutants that varied in phenolic compound content and exhibited stable inheritance of the mutated characteristics from the M_7 to M_9 generations were selected. Radiation-generated mutant genotypes were grown by the Radiation Breeding Research Team at the Advanced Radiation Technology Institute of the Korea Atomic Energy Research Institute (35°51′09″ N, 126°83′41″ E). The seeds were planted in plots (3 × 6 m) and row spacing of 20 and 40 cm, respectively. Fertilizer (N:P:K 4:2:2 *w*/*w*/*w*) was applied to 600 kg·ha^{-1} at pre-sowing, and the plants were not fertilized after sowing. Seed yield was surveyed on 95 mutants and original cultivars during three generations (M_7 to M_9). Young leaves were sampled from the two original cultivars (Youngsan and Tammi) and 95 rapeseed mutants. Genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

2.2. Library Construction and Genotyping-by-Sequencing

The construction of GBS libraries involved the use of the restriction enzyme *ApeKI* (5′-GCWGC-3′) following a modified protocol from a previously published study [18]. The oligonucleotides for the top and bottom strands of each barcode adapter and common adapter were diluted in TE (50 µM each) and annealed using a thermocycler. In total, 100 ng/µL of DNA samples were added to wells containing the adapter. The samples were then digested with *ApeKI* (New England Biolabs, Ipswich, MA, USA) overnight at 75 °C. The digested DNA samples, each with a different barcode adapter, were combined (5 µL each) and purified using a commercial kit (QIAquick PCR Purification Kit; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Amplification of the restriction fragments from each library was performed in 50 µL volumes using HerculaseII Fusion DNA Polymerase (Agilent, CA, USA) and primers (A) 5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T-3′ and (B) 5′-CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T-3′. The amplified sample pools were then used as the sequencing "library" and were sequenced on the Illumina HiSeq 2000 platform by SEEDERS Co. (Daejeon, Republic of Korea).

2.3. Sequence Preprocessing and Alignment to Reference Genome Sequence

The demultiplexing process involved the use of the barcode sequence, followed by adapter removal and sequence quality trimming. Adapter trimming was carried out using Cutadapt (Version 1.8.3) [23], and sequence quality trimming was performed using the

DynamicTrim and LengthSort features of the SolexaQA program (V.1.13) [17]. The DynamicTrim tool trimmed low-quality bases at the ends of short reads based on the Phred score and produced high-quality cleaned reads. LengthSort removed excessive base cuts made by DynamicTrim and kept only reads with a Phred score of ≥20 and a length of ≥25 bp. The cleaned reads were then mapped to the reference genome sequence of *Brassica napus* V5.1 using BWA (0.6.1-r104) [24]. The mapping process was a preliminary step for detecting raw SNPs (In/Del) in the *B. napus* Ver. 5.1 (http://www.genoscope.cns.fr/brassicanapus accessed on 10 April 2023) and sequenced samples. A SAM file was generated using default values, with the exception of the following options: seed length (−*l*) of 30, maximum differences in the seed (−*k*) of 1, number of threads (−*t*) of 16, mismatch penalty (−*M*) of 6, gap opening penalty (−*O*) of 15, and gap extension penalty (−*E*) of 8. The experiment was repeated to ensure accuracy.

2.4. Raw SNP Detection and Consensus Sequence Extraction

The cleaned reads were mapped to the standard genome sequence, and the resulting SAM files were used to detect raw single nucleotide polymorphisms (SNPs) using SAMtools (0.1.16) [25] and to extract consensus sequences. Before SNP detection, the SNPs were validated using an in-house script developed by SEEDERS [26]. Raw SNP detection was performed with default values, with the exception of the following options: minimum mapping quality for SNPs (−*Q*) of 30, minimum mapping quality for gaps (−*q*) of 15, minimum read depth (−*d*) of 3, minimum indel score for nearby SNP filtering (−*G*) of 30, SNPs within 15 bp of a gap to be filtered (−*w*) of 15, window size for filtering dense SNPs (−*W*) of 30, and maximum read depth (−*D*) of 489.

2.5. Generate SNP Matrix

An integrated SNP matrix was generated from the samples to analyze the SNPs among the objects of interest. The process involved the creation of a list of unions using raw SNP positions obtained by comparing each sample with a reference genome (Brassica napus V. 5.1). Non-SNP loci were filled in from the consensus sequence of each sample. The final SNP matrix was generated by filtering out miscalled SNP positions using comparisons among the samples. SNPs were then divided into homozygous (read depth ≥ 90%), heterozygous (40–60%), and other (undistinguishable by type) groups based on their positions [21]. The SNP positions were then classified into intergenic or genic regions of the reference genome, with the genic region further classified into CDS or intron regions. To identify the SNPs that were unique to each mutant line, the common SNP in the original cultivars 'Youngsan' and 'Tammi' was first selected in the integrated SNP matrix, followed by the selection of polymorphic SNPs by comparing the common SNP of the original cultivar with the base sequence of each mutant. Finally, the SNP loci of each mutant line were integrated to secure the SNP locus of the union for subsequent gene ontology and functional analysis.

2.6. Ultra-High-Performance Liquid Chromatography (UPLC) and Total Phenolic Content Analysis

The phenolic compounds present in the fully mature seeds were analyzed using UPLC [7]. Rapeseed seed (10 g) was extracted using a mixture of methanol and water (80:20 *v*/*v*) and filtered through a polyvinylidene fluoride syringe filter. The UPLC system was equipped with an XR-ODS column and a C18 guard column, and the mobile phase consisted of solvent A (0.1% trifluoroacetic acid in distilled water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). The phenolic compounds were separated using a gradient of solvent B, and detection was performed at 280 nm. The peaks in the HPLC chromatogram were identified based on spectra, standards, and data from the literature. For quantification, calibration curves of these standards were prepared from measurements at five concentrations (25, 50, 75, 100, and 125 mg/mL). The linear equation for sinapine (Sigma, St. Louis, MO, USA) was y = 77901X + 69457. The calibration curves were linear with a high correlation coefficient (r^2 = 0.9932). Seeds were collected from the fully mature

periods for each mutant line. The samples were quantified using sinapine, and the results were based on three biological replicates for each sample.

The total phenolic content (TPC) was determined using the Folin–Ciocalteu colorimetric method [27]. A small quantity (0.2 mL) of each extract and 1.5 mL of Folin–Ciocalteu reagent (20% *v*/*v*) were mixed thoroughly. Next, 4 mL of Na_2CO_3 (7%) was added, then made up to 10 mL with water. The mixture was kept in the dark at room temperature for 90 min. The absorbance was then measured at 760 nm using a UV-spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). TPC was calculated using a calibration curve of sinapic acid (Sigma, USA). Calibration curves of these standards were prepared from measurements at seven concentrations (25, 50, 75, 100, 125, 150, and 200 mg/mL). The linear equation for sinapic acid was $y = 0.101X - 0.0181$. The calibration curves were linear with a high correlation coefficient ($r^2 = 0.9974$).

2.7. Association and Networking Study

In the association analysis, the 70,208 union SNP dataset was used, and the FarmCPU model was utilized in the genomic association and prediction integrated tool (GAPIT) R package [28]. The Q general linear model (GLM) was performed on the chosen Q-matrix derived from STRUCTURE to determine the experiment-wise *p*-value for each marker's significance. The next step was to create a Q-mixed linear model (Q-MLM) method with a kinship matrix. The significance of SNPs was determined based on a *p*-value < 0.0001 with corresponding −log10 (*p*) values of 4.0 for the significant threshold. The annotated genes that were selected as significant SNPs were evaluated for their probable interactions, co-expression, predictions, co-localization, and shared proteins to define gene functions using GeneMANIA (https://genemania.org/ accessed on 2 May 2023).

3. Results

3.1. Genotyping-by-Sequencing of Rapeseed Genotypes

A GBS analysis was conducted on 95 mutant lines and two original cultivars of rapeseed using the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA). A summary of the sequence data generated from the 95 rapeseed mutant lines and the two original cultivars is shown in Table 1 and Table S1. A total of 1088 million reads were generated, with an average of 11 million reads per sample. After removing low-quality sequences, there were 967,244,884 clean reads, with an average of 9.9 million reads per plant sample. The total length of the clean reads ranged from 140,097,724 bp to 3,013,306,105 bp, with an average read length of 1,112,595,675 bp. The total number of mapped reads was 740,184,076 for all lines, with an average of 7,630,764 reads per sample. The proportion of reads mapped to the reference genome sequence ranged from 27.79% to 91.10%, with an average of 75.14%. The total length of the mapped region was 1,765,446,134 bp, with an average of 18,200,476 bp per sample. The mapped region covered approximately 2.14% of the reference genome sequence with an average depth of 15.82×.

Table 1. Summary of GBS data and alignment to the reference genome sequence.

	Total	**Average/Plant**
	Raw data	
Reads	1,088,590,054	11,222,578
Bases (bp)	164,377,098,154	1,694,609,259
	After trimming	
Reads	967,244,884	9,971,597
Bases (bp)	107,921,780,499	1,112,595,675
	Mapped reads on reference genome [1]	
Mapped reads	740,184,076	7,630,764
Bases (bp)	1,765,446,134	18,200,476

Table 1. *Cont.*

	Total	Average/Plant
Reference genome coverage (%)		2.14
Reference genome depth		15.82

[1] Reference genome sequence: *Brassica napus* V5.1 (http://www.genoscope.cns.fr/brassicanapus accessed on 10 April 2023).

3.2. Identification of SNPs

The distribution of union SNPs on rapeseed chromosomes is shown in Figure 1. The number of union SNPs on rapeseed chromosomes ranged from 1850 (chromosome A02) to 4638 (chromosome C01). Chromosomes C01, C02, C03, and C08 had more union SNPs than did the other chromosomes. Chromosome A09 had the highest number (2887) of union SNPs on genic regions.

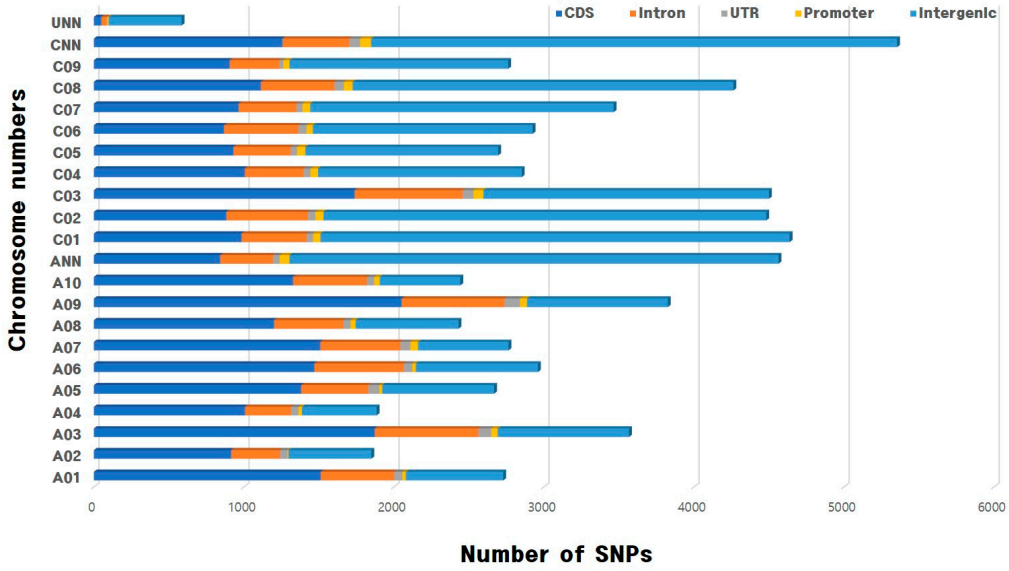

Figure 1. Chromosomal distribution of 70,208 union SNPs identified using GBS in 95 mutant lines obtained with gamma-ray irradiation treatment. Reference genome sequence: *Brassica napus* V.5.1 (http://www.genoscope.cns.fr/brassicanapus accessed on 10 April 2023).

The SNPs for each line were first selected by comparing the union SNPs in each matrix position between the sample and the reference genome sequence (Table S2). A total of 3,196,318 SNPs were identified, with the majority being homozygous (2,025,206). The highest number of SNPs (51,816) was recorded in the Tm8-10 mutant line, and the lowest number (10,717) was recorded in the Y6-203 mutant line.

Polymorphic SNPs were identified by comparing the common SNPs in the original cultivar with the base sequences of the mutant lines, wherein any changes were considered to have resulted from mutation (Table S3; Supplementary Materials File S1). The total number of polymorphic SNPs in each line ranged from 417 (Y6-322) to 11,256 (Y10-32), with an average of 6654. The number of homozygous SNPs in each rapeseed mutant line ranged from 210 (Y6-322) to 10,357 (Y10-12), with an average of 5318 per mutant line. A collection of 70,208 SNPs without overlaps was constructed (Table S4). Of these, 32,276 were located in intergenic regions, and 37,932 were located in genic regions, encompassing 16,550 genes. Among the SNPs in genic regions, 25,807 were located in gene coding sequences, 900 in gene promoter regions, 10,031 in introns, and 1194 in untranslated regions.

3.3. Phenolic Compound Content and Seed Yield

The phenolic compound contents in the 95 mutant lines and the two original cultivars are shown in Table 2 and Figure 2. Seven main phenolic compounds were identified: dihex-

ose (peak 1, tR 4.6; $C_{12}H_{22}O_{11}$), progoitrin (peak 2, tR 6.5; $C_{11}H_{19}NO_{10}S_2$), *trans*-sinapine 1 (peak 3, tR 16.1; $C_{16}H_{24}NO_5$), *trans*-sinapine 2 (peak 4, tR 16.4; $C_{16}H_{24}NO_5$), sinapine(4-*O*-8′)guaiacyl (peak 5, tR 17.7; $C_{25}H_{24}O_{12}$), sinapoyl malate (peak 6, tR 20.3; $C_{15}H_{16}O_9$), and methyl sinapate (peak 7, tR 25.4; $C_{12}H_{14}O_5$) (Figure 3 and Table S5). The contents of dihexose, progoitrin, *trans*-sinapine 1, *trans*-sinapine 2, sinapine(4-*O*-8′)guaiacyl, sinapoyl malate, and methyl sinapate were 0.70 ± 0.29, 0.15 ± 0.06, 16.03 ± 0.04, 17.65 ± 2.00, 0.14 ± 0.08, 0.94 ± 0.11, and 0.05 ± 0.01 mg·g^{-1}, respectively, in 'Youngsan'. The contents of dihexose, progoitrin, *trans*-sinapine 1, *trans*-sinapine 2, sinapine(4-*O*-8′)guaiacyl, sinapoyl malate, and methyl sinapate were 0.21 ± 0.04, 0.08 ± 0.01, 16.03 ± 0.29, 7.64 ± 1.63, 22.22 ± 1.48, 0.12 ± 0.03, 1.08 ± 0.27, and 0.09 ± 0.03 mg·g^{-1}, respectively, in 'Tammi'. Sinapine was the major (91.2–99.2%) phenolic compound in all genotypes. Significant difference in sinapine content were observed among mutant lines. The *trans*-sinapine 1 content of the mutant lines ranged from 1.86 ± 0.36 mg·g^{-1} to 25.85 ± 1.32 mg·g^{-1}. The *trans*-sinapine 1 content was highest in Tm10-3 and lowest in Tm10-5EF. The *trans*-sinapine 2 content was highest in Tm7M-1 (25.67 ± 1.22 mg·g^{-1}) and lowest in Tm10-3 (1.11 ± 0.25 mg·g^{-1}). The total contents of the seven main phenolic compounds ranged from 46.03 mg·g^{-1} (in Y6-81a) to 8.31 mg·g^{-1} (in Y6-29-30). Dihexose, progoitrin, sinapine(4-*O*-8)guaiacyl, sinapoyl malate, and methyl sinapate were present in relatively small amounts in all genotypes. The progoitrin (glucosinolates) content in the mutant lines ranged from 0.03 ± 1.22 mg·g^{-1} in Tm10-9EF to 0.50 ± 0.05 mg·g^{-1} in Tm6-7. Among the mutant lines, Y-6-81a showed higher levels of dihexose (1.48 mg·g^{-1}), progoitrin (0.40 mg·g^{-1}), sinapine(4-*O*-8′)guaiacyl (0.21 mg·g^{-1}), cyclic spermidine-alkaloid (0.62 mg·g^{-1}), and methyl sinapate (0.29 mg·g^{-1}).

Table 2. Contents of phenolic compounds in seed extracts from 97 different rapeseed genotypes.

No.	Line Names	Dihexose	Progoitrin	*trans*-Sinapine 1	*trans*-Sinapine 2	Sinapine (4-O-8′) Guaiacyl	Sinapoyl Malate	Methyl Sinapate	Total
1	Youngsan	0.70 ± 0.29	0.15 ± 0.06	16.03 ± 0.04	17.65 ± 2.00	0.14 ± 0.08	0.94 ± 0.11	0.05 ± 0.01	35.66 ± 2.69
2	Y6-3	0.49 ± 0.21	0.24 ± 0.07	13.25 ± 2.00	11.77 ± 1.04	0.15 ± 0.02	0.22 ± 0.05	0.05 ± 0.01	26.18 ± 3.50
3	Y6-12	0.56 ± 0.20	0.18 ± 0.02	14.00 ± 2.04	12.50 ± 1.13	0.16 ± 0.01	0.27 ± 0.06	0.06 ± 0.02	27.73 ± 3.58
4	Y6-11	0.52 ± 0.23	0.13 ± 0.07	18.46 ± 0.30	22.89 ± 0.88	0.16 ± 0.06	0.30 ± 0.06	0.04 ± 0.01	42.50 ± 1.71
5	Y6-21	0.38 ± 0.13	0.16 ± 0.06	12.41 ± 1.04	09.11 ± 0.55	0.21 ± 0.04	0.04 ± 0.01	0.18 ± 0.07	22.48 ± 1.99
6	Y6-9EF1	0.60 ± 0.24	0.13 ± 0.07	14.74 ± 0.74	14.18 ± 1.47	0.20 ± 0.06	0.68 ± 0.13	0.04 ± 0.01	30.57 ± 2.82
7	Y6-9EF2	0.67 ± 0.30	0.26 ± 0.05	14.87 ± 0.25	13.54 ± 1.40	0.18 ± 0.03	0.32 ± 0.06	0.04 ± 0.01	29.88 ± 2.21
8	Y6-81a	1.48 ± 0.66	0.40 ± 0.00	18.07 ± 1.13	24.77 ± 1.45	0.21 ± 0.03	0.81 ± 0.15	0.29 ± 0.08	46.03 ± 3.71
9	Y6-82a	1.23 ± 0.55	0.36 ± 0.01	15.70 ± 0.88	16.85 ± 1.65	0.23 ± 0.04	0.69 ± 0.12	0.25 ± 0.07	35.31 ± 3.43
10	Y6-171	1.11 ± 0.48	0.34 ± 0.02	13.82 ± 0.24	14.19 ± 1.55	0.20 ± 0.02	0.63 ± 0.12	0.22 ± 0.07	30.51 ± 2.60
11	Y6-181	0.40 ± 0.13	0.15 ± 0.05	13.72 ± 0.88	10.19 ± 0.82	0.08 ± 0.00	0.34 ± 0.10	0.12± 0.04	25.01 ± 2.01
12	Y6-192	0.43 ± 0.16	0.15 ± 0.05	11.25 ± 1.12	09.12 ± 0.57	0.07 ± 0.00	0.34 ± 0.09	0.15 ± 0.05	21.51 ± 2.04
13	Y6-203	0.29 ± 0.10	0.14 ± 0.06	12.06 ± 0.58	09.78 ± 0.72	0.17 ± 0.03	0.02 ± 0.01	0.12 ± 0.04	22.59 ± 1.54
14	Y6-221	0.33 ± 0.12	0.15 ± 0.05	10.95 ± 0.55	08.72 ± 0.50	0.18 ± 0.05	0.02 ± 0.01	0.13 ± 0.04	20.49 ± 1.31
15	Y6-241	0.32 ± 0.12	0.15 ± 0.05	10.81 ± 0.03	08.85 ± 0.53	0.19 ± 0.05	0.02 ± 0.00	0.12 ± 0.04	20.47 ± 0.83
16	Y6-242	0.28 ± 0.10	0.14 ± 0.06	12.77 ± 0.23	10.14 ± 0.82	0.16 ± 0.03	0.02 ± 0.01	0.14 ± 0.04	23.64 ± 1.29
17	Y6-243	0.29 ± 0.10	0.14 ± 0.05	11.63 ± 1.47	09.39 ± 0.64	0.14 ± 0.03	0.02 ± 0.00	0.11 ± 0.03	21.72 ± 2.34
18	Y6-251	0.90 ± 0.40	0.27 ± 0.03	13.43 ± 1.24	10.93 ± 1.05	0.28 ± 0.08	0.01 ± 0.00	0.11 ± 0.03	25.93 ± 2.83
19	Y6-262	0.40 ± 0.12	0.16 ± 0.04	11.94 ± 0.17	10.17 ± 0.86	0.19 ± 0.05	0.01 ± 0.00	0.14 ± 0.04	23.01 ± 1.28
20	Y6-271	0.33 ± 0.11	0.10 ± 0.02	09.74 ± 1.40	08.46 ± 0.48	0.13 ± 0.03	0.02 ± 0.01	0.08 ± 0.03	18.87 ± 2.07
21	Y6-292	0.25 ± 0.06	0.13 ± 0.05	10.97 ± 1.22	09.28 ± 0.77	0.07 ± 0.01	0.05 ± 0.01	0.14 ± 0.06	20.89 ± 2.20
22	Y6-294	0.29 ± 0.09	0.13 ± 0.05	12.69 ± 0.08	10.62 ± 1.02	0.11 ± 0.04	0.01 ± 0.00	0.18 ± 0.07	24.03 ± 1.34
23	Y6-321	0.30 ± 0.11	0.09 ± 0.02	12.08 ± 1.45	09.54 ± 0.88	0.13 ± 0.02	0.04 ± 0.01	0.10 ± 0.04	22.27 ± 2.54
24	Y6-322	0.44 ± 0.17	0.10 ± 0.01	16.71 ± 1.53	13.38 ± 1.61	0.15 ± 0.03	0.04 ± 0.01	0.17 ± 0.06	30.99 ± 3.43
25	Y6-351	0.46 ± 0.22	0.09 ± 0.04	12.26 ± 0.02	12.78 ± 1.35	0.11 ± 0.02	0.19 ± 0.04	0.04 ± 0.01	25.94 ± 1.70
26	Y8-18-12	0.33 ± 0.13	0.11 ± 0.01	10.64 ± 1.65	09.07 ± 0.67	0.08 ± 0.02	0.03 ± 0.01	0.12 ± 0.04	20.37 ± 2.53
27	Y8-18-13	0.31 ± 0.11	0.10 ± 0.02	09.82 ± 1.63	08.41 ± 0.48	0.06 ± 0.02	0.04 ± 0.01	0.08 ± 0.02	18.82 ± 2.28
28	Y8-18-14	0.41 ± 0.17	0.13 ± 0.00	11.24 ± 0.22	09.69 ± 0.77	0.07 ± 0.02	0.04 ± 0.01	0.13 ± 0.06	21.72 ± 1.25
29	Y8-18-21	0.31 ± 0.11	0.09 ± 0.02	10.01 ± 1.55	08.37 ± 0.50	0.07 ± 0.02	0.02 ± 0.01	0.10 ± 0.03	18.98 ± 2.24
30	Y8-18-22	0.35 ± 0.13	0.11 ± 0.02	13.04 ± 1.33	10.94 ± 1.08	0.09 ± 0.02	0.03 ± 0.01	0.16 ± 0.07	24.71 ± 2.65
31	Y8-18-23	0.25 ± 0.08	0.09 ± 0.02	07.66 ± 0.60	06.74 ± 0.18	0.05 ± 0.02	0.02 ± 0.01	0.11 ± 0.04	14.91 ± 0.95
32	Y10-12	0.37 ± 0.13	0.16 ± 0.04	10.26 ± 0.82	08.99 ± 0.77	0.09 ± 0.03	0.06 ± 0.02	0.18 ± 0.07	20.11 ± 1.88
33	Y10-31	0.23 ± 0.08	0.09 ± 0.02	09.57 ± 0.22	08.11 ± 0.51	0.07 ± 0.03	0.03 ± 0.01	0.14 ± 0.05	18.24 ± 0.93
34	Y10-32	0.31 ± 0.11	0.09 ± 0.02	12.85 ± 0.69	10.45 ± 1.31	0.14 ± 0.04	0.05 ± 0.01	0.17 ± 0.08	24.06 ± 2.28
35	Y10-3-21	0.33 ± 0.11	0.14 ± 0.04	07.20 ± 0.57	06.05 ± 0.07	0.06 ± 0.01	0.04 ± 0.01	0.07 ± 0.02	13.89 ± 0.84
36	Y10-1b2	0.43 ± 0.18	0.12 ± 0.00	10.15 ± 0.12	08.20 ± 0.51	0.19 ± 0.05	0.02 ± 0.00	0.11 ± 0.04	19.22 ± 0.91
37	Y6-18-2	0.27 ± 0.09	0.09 ± 0.03	09.79 ± 0.49	07.94 ± 0.49	0.04 ± 0.01	0.02 ± 0.00	0.11 ± 0.03	18.25 ± 1.14
38	Y6-81b	0.40 ± 0.14	0.15 ± 0.04	07.39 ± 0.72	06.18 ± 0.09	0.07 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	14.27 ± 1.03
39	Y6-191	0.37 ± 0.12	0.10 ± 0.02	13.00 ± 0.23	09.84 ± 0.95	0.07 ± 0.03	0.02 ± 0.01	0.14 ± 0.05	23.54 ± 1.41

Table 2. *Cont.*

No.	Line Names	Dihexose	Progoitrin	*trans*-Sinapine 1	*trans*-Sinapine 2	Sinapine (4-O-8′) Guaiacyl	Sinapoyl Malate	Methyl Sinapate	Total
40	Y8-18-11	0.25 ± 0.10	0.09 ± 0.02	08.53 ± 0.57	07.26 ± 0.32	0.06 ± 0.01	0.02 ± 0.01	0.13 ± 0.05	16.35 ± 1.08
41	Y6-201	0.51 ± 0.20	0.14 ± 0.01	07.18 ± 0.50	06.28 ± 0.13	0.15 ± 0.04	0.02 ± 0.01	0.08 ± 0.02	14.37 ± 0.91
42	Y6-202	0.20 ± 0.07	0.08 ± 0.02	07.05 ± 0.07	05.78 ± 0.06	0.08 ± 0.02	0.02 ± 0.01	0.11 ± 0.03	13.32 ± 0.27
43	Y6-183	0.46 ± 0.15	0.10 ± 0.02	09.16 ± 0.58	07.80 ± 0.50	0.04 ± 0.02	0.02 ± 0.01	0.14 ± 0.04	17.73 ± 1.32
44	Y6-252	0.37 ± 0.12	0.10 ± 0.01	09.76 ± 0.53	08.12 ± 0.54	0.15 ± 0.04	0.01 ± 0.00	0.07 ± 0.02	18.59 ± 1.26
45	Y6-291	0.29 ± 0.09	0.10 ± 0.02	06.85 ± 0.05	06.69 ± 0.26	0.04 ± 0.01	0.10 ± 0.02	0.11 ± 0.03	14.17 ± 0.49
46	Y6-222	0.37 ± 0.15	0.10 ± 0.01	09.73 ± 0.47	08.30 ± 0.62	0.13 ± 0.03	0.02 ± 0.01	0.12 ± 0.05	18.77 ± 1.34
47	Y6-82b	1.41 ± 0.70	0.29 ± 0.08	13.32 ± 0.82	16.32 ± 2.64	0.09 ± 0.03	0.71 ± 0.16	0.25 ± 0.07	32.39 ± 4.50
48	Y6-22	0.41 ± 0.17	0.11 ± 0.01	08.91 ± 0.35	07.69 ± 0.55	0.17 ± 0.03	0.02 ± 0.01	0.14 ± 0.06	17.44 ± 1.18
49	Y6172	0.32 ± 0.11	0.10 ± 0.01	06.41 ± 0.54	05.50 ± 0.11	0.05 ± 0.01	0.02 ± 0.00	0.06 ± 0.02	12.47 ± 0.80
50	Y6-29-30	0.39 ± 0.13	0.15 ± 0.03	04.31 ± 0.64	04.01 ± 0.16	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	08.96 ± 0.99
51	Tammi	0.21 ± 0.04	0.08 ± 0.01	07.64 ± 1.63	22.22 ± 1.48	0.12 ± 0.03	1.08 ± 0.27	0.09 ± 0.03	31.45 ± 3.50
52	Tm2M-1	0.30 ± 0.06	0.05 ± 0.01	03.67 ± 0.66	17.14 ± 1.32	0.05 ± 0.02	0.29 ± 0.07	0.04 ± 0.01	21.54 ± 2.15
53	Tm3M-1	0.69 ± 0.13	0.45 ± 0.19	03.36 ± 0.55	17.96 ± 1.40	0.01 ± 0.00	0.35 ± 0.09	0.07 ± 0.03	22.89 ± 2.39
54	Tm3M-2	0.07 ± 0.01	0.08 ± 0.01	18.85 ± 1.74	12.29 ± 2.72	0.05 ± 0.01	0.30 ± 0.08	0.02 ± 0.01	31.65 ± 4.58
55	Tm4M-1	0.48 ± 0.04	0.35 ± 0.13	04.50 ± 0.77	19.64 ± 1.35	0.07 ± 0.03	0.86 ± 0.20	0.07 ± 0.02	25.97 ± 2.54
56	Tm4M-2	0.34 ± 0.03	0.10 ± 0.01	04.15 ± 0.67	19.71 ± 1.55	0.07 ± 0.03	0.41 ± 0.10	0.06 ± 0.02	24.84 ± 2.41
57	Tm7M-1	0.57 ± 0.12	0.41 ± 0.09	07.09 ± 0.97	25.67 ± 1.22	0.12 ± 0.04	1.01 ± 0.26	0.31 ± 0.09	35.18 ± 2.79
58	Tm7M-2	0.52 ± 0.11	0.37 ± 0.07	04.39 ± 1.14	19.56 ± 1.30	0.09 ± 0.03	0.78 ± 0.20	0.24 ± 0.07	25.94 ± 2.91
59	Tm6-1	0.03 ± 0.01	0.08 ± 0.01	11.41 ± 1.82	12.92 ± 2.77	0.07 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	24.55 ± 4.65
60	Tm6-2	0.06 ± 0.00	0.13 ± 0.02	06.34 ± 0.99	11.16 ± 2.59	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	17.78 ± 3.63
61	Tm6-3	0.05 ± 0.00	0.14 ± 0.01	03.80 ± 0.50	12.30 ± 2.88	0.05 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	16.38 ± 3.43
62	Tm6-4	0.54 ± 0.10	0.48 ± 0.00	04.82 ± 1.14	21.71 ± 0.90	0.10 ± 0.03	0.87 ± 0.22	0.27 ± 0.08	28.79 ± 2.47
63	Tm6-6	0.21 ± 0.03	0.11 ± 0.00	04.79 ± 0.94	24.41 ± 1.38	0.07 ± 0.02	0.38 ± 0.09	0.01 ± 0.00	29.98 ± 2.47
64	Tm6-7	0.43 ± 0.07	0.50 ± 0.05	04.54 ± 0.80	11.23 ± 2.65	0.05 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	16.80 ± 3.61
65	Tm6-8	0.39 ± 0.06	0.35 ± 0.04	03.10 ± 0.35	11.21 ± 2.65	0.04 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	15.13 ± 3.12
66	Tm6-10	0.03 ± 0.01	0.07 ± 0.00	02.77 ± 0.43	12.22 ± 2.71	0.04 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	15.16 ± 3.18
67	Tm6-12	0.55 ± 0.09	0.40 ± 0.03	04.86 ± 0.98	16.93 ± 1.01	0.07 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	22.85 ± 2.15
68	Tm6-13	0.13 ± 0.01	0.08 ± 0.00	04.06 ± 0.81	13.70 ± 2.66	0.06 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	18.09 ± 3.52
69	Tm8-2	0.10 ± 0.04	0.07 ± 0.01	02.85 ± 0.54	11.25 ± 2.53	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	14.37 ± 3.14
70	Tm8-3	0.04 ± 0.00	0.06 ± 0.00	09.94 ± 2.35	11.65 ± 2.66	0.05 ± 0.01	0.07 ± 0.02	0.03 ± 0.01	21.84 ± 5.06
71	Tm8-4	0.13 ± 0.02	0.06 ± 0.01	13.21 ±3.34	13.43 ± 2.66	0.09 ± 0.02	0.05 ± 0.01	0.04 ± 0.01	27.01 ± 6.07
72	Tm8-5	0.03 ± 0.01	0.06 ± 0.01	20.81 ± 1.32	11.85 ± 2.86	0.07 ± 0.02	0.06 ± 0.02	0.02 ± 0.01	32.91 ± 4.23
73	Tm8-6	0.09 ± 0.02	0.07 ± 0.00	22.15 ± 1.57	18.04 ± 0.30	0.10 ± 0.02	0.06 ± 0.02	0.04 ± 0.01	40.55 ± 1.94
74	Tm8-7	0.14 ± 0.03	0.08 ± 0.01	02.74 ± 0.69	17.10 ± 1.03	0.09 ± 0.02	0.25 ± 0.06	0.01 ± 0.00	20.40 ± 1.84
75	Tm8-8	0.09 ± 0.00	0.07 ± 0.00	04.85 ± 1.03	11.00 ± 2.70	0.07 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	16.17 ± 3.77
76	Tm8-10	0.07 ± 0.03	0.06 ± 0.00	04.34 ± 0.86	10.71 ± 2.47	0.05 ± 0.01	0.06 ± 0.02	0.02 ± 0.01	15.31 ± 3.39
77	Tm8-11	0.17 ± 0.04	0.31 ± 0.04	05.54 ± 1.20	12.38 ± 2.43	0.07 ± 0.02	0.05 ± 0.01	0.04 ± 0.01	18.55 ± 3.75
78	Tm8-12	0.08 ± 0.02	0.06 ± 0.00	04.74 ± 1.17	10.31 ± 2.38	0.05 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	15.30 ± 3.60
79	Tm8-13	0.18 ± 0.01	0.27 ± 0.03	07.81 ± 0.38	15.66 ± 0.69	0.08 ± 0.02	0.04 ± 0.01	0.05 ± 0.02	24.09 ±1.16
80	Tm8-14	0.08 ± 0.00	0.05 ± 0.01	04.38 ± 1.09	07.92 ± 2.14	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	12.53 ± 3.27
81	Tm8-15	0.17 ± 0.01	0.28 ± 0.02	08.00 ± 1.97	10.37 ± 2.43	0.08 ± 0.02	0.08 ± 0.02	0.05 ± 0.02	19.03 ±4.49
82	Tm8-16	0.09 ± 0.00	0.06 ± 0.00	03.30 ± 0.79	09.37 ± 2.33	0.12 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	13.02 ± 3.16
83	Tm8-17	0.17 ± 0.01	0.31 ± 0.00	08.42 ± 1.18	11.47 ± 2.51	0.26 ± 0.04	0.07 ± 0.02	0.06 ± 0.02	20.74 ± 3.78
84	Tm10-1	0.08 ± 0.01	0.06 ± 0.01	03.28 ± 0.73	07.32 ± 2.03	0.10 ± 0.02	0.06 ± 0.01	0.02 ± 0.01	10.92 ± 2.81
85	Tm10-1St	0.16 ± 0.02	0.30 ± 0.03	05.39 ± 0.42	09.93 ± 2.28	0.34 ± 0.06	0.04 ± 0.00	0.03 ± 0.01	16.20 ± 2.82
86	Tm10-1Lin	0.13 ± 0.01	0.16 ± 0.03	07.08 ± 0.83	09.06 ± 2.15	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	16.52 ± 3.05
87	Tm10-2	0.09 ± 0.02	0.07 ± 0.00	02.12 ± 0.33	07.52 ± 2.04	0.12 ± 0.02	0.04 ± 0.01	0.02 ± 0.01	09.98 ± 2.43
88	Tm10Oel	0.14 ± 0.01	0.22 ± 0.03	11.30 ± 1.75	12.54 ± 2.19	0.13 ± 0.02	0.02 ± 0.01	0.05 ± 0.02	24.40 ± 4.03
89	Tm10-3	0.08 ± 0.00	0.05 ± 0.00	25.85 ± 1.32	01.11 ± 0.25	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	27.21 ± 1.60
90	Tm10-4EF	0.51 ± 0.08	0.20 ± 0.04	03.11 ± 0.58	07.95 ± 2.03	0.30 ± 0.06	0.03 ± 0.01	0.02 ± 0.01	12.13 ± 2.81
91	Tm10-5EF	0.10 ± 0.01	0.08 ± 0.03	01.86 ± 0.36	06.93 ± 1.92	0.14 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	09.18 ± 2.35
92	Tm10-6EF	0.08 ± 0.01	0.04 ± 0.00	08.02 ± 1.80	10.28 ± 2.09	0.10 ± 0.03	0.03 ± 0.01	0.05 ± 0.02	18.60 ± 3.95
93	Tm10-7EF	0.09 ± 0.01	0.06 ± 0.01	04.51 ± 0.26	10.40 ± 2.13	0.31 ± 0.06	0.02 ± 0.00	0.02 ± 0.01	15.39 ± 2.47
94	Tm10-8EF	0.08 ± 0.01	0.04 ± 0.00	08.61 ± 0.54	08.03 ± 2.03	0.06 ± 0.01	0.13 ± 0.03	0.04 ± 0.01	16.99 ± 2.64
95	Tm10-9EF	0.28 ± 0.10	0.03 ± 0.01	04.36 ± 0.30	10.81 ± 2.00	0.27 ± 0.06	0.03 ± 0.01	0.04 ± 0.01	15.83 ± 2.49
96	Tm10-10EF	0.06 ± 0.01	0.22 ± 0.02	04.88 ± 0.27	20.93 ± 0.87	0.53 ± 0.12	0.80 ± 0.16	0.27 ± 0.07	27.68 ± 1.53
97	Tm10-11EF	0.31 ± 0.11	0.04 ± 0.01	11.82 ± 0.83	09.59 ± 2.16	0.33 ± 0.08	0.05 ± 0.01	0.05 ± 0.02	22.20 ± 3.21

Values (mean ± SD) of extracts analyzed individually in triplicate.

The seed yields of the 95 mutant lines and the two original cultivars are shown in Figure 4. The seed yields of 'Youngsan' and 'Tammi' were 2856.7 ± 27.5 kg·ha^{-1} and 3420.0 ± 33.5 kg·ha^{-1}, respectively. Significant difference in seed yield were observed among the mutant lines. The seed yield of the mutant lines ranged from 2333.3 ± 20.2 kg·ha^{-1} (in Y6-21) to 3602.7 ± 15.7 kg·ha^{-1} (in TM6-3) with an average of 2943.6 ± 19.4 kg·ha^{-1}.

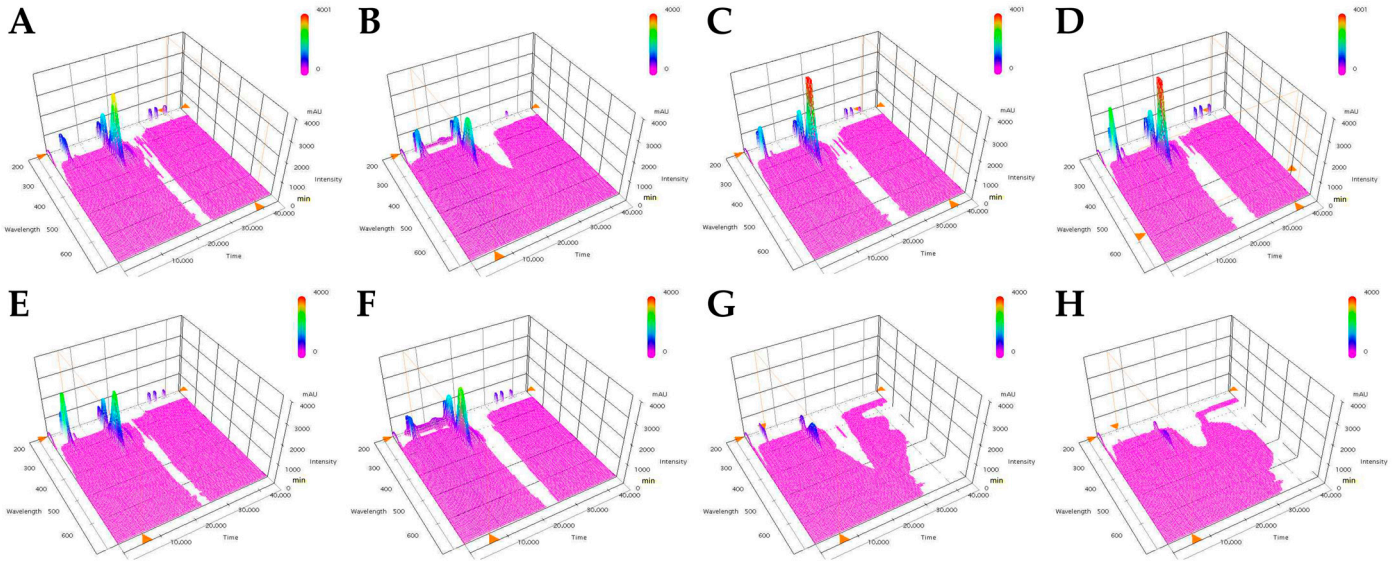

Figure 2. Three-dimensional chromatogram from UPLC analysis of different rapeseed genotypes. (**A**): 'Youngsan', (**B**): 'Tammi', (**C**): Y6-11, (**D**): Y6-81a, (**E**): Tm7M-1, (**F**): Tm10-3, (**G**): Tm10-5EF, and (**H**): Y6-29-30.

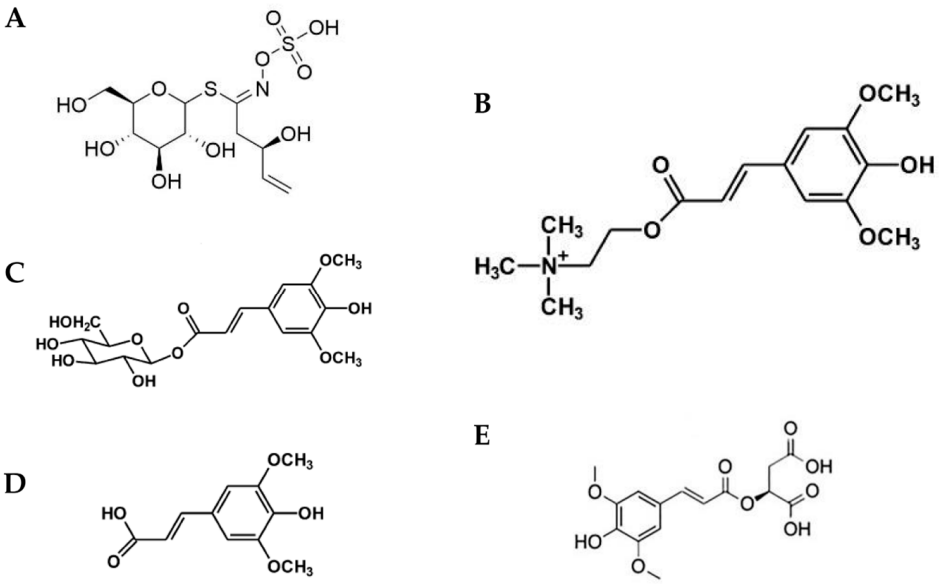

Figure 3. Chemical structure of major glucosinolate (**A)** and phenolic (**B**–**E**) in rapeseed seed. (**A**): Progoitrin, (**B**): sinapine, (**C**): sinapine(4-*O*-8′)guaiacyl, (**D**): sinapoyl malate, and (**E**): sinapate.

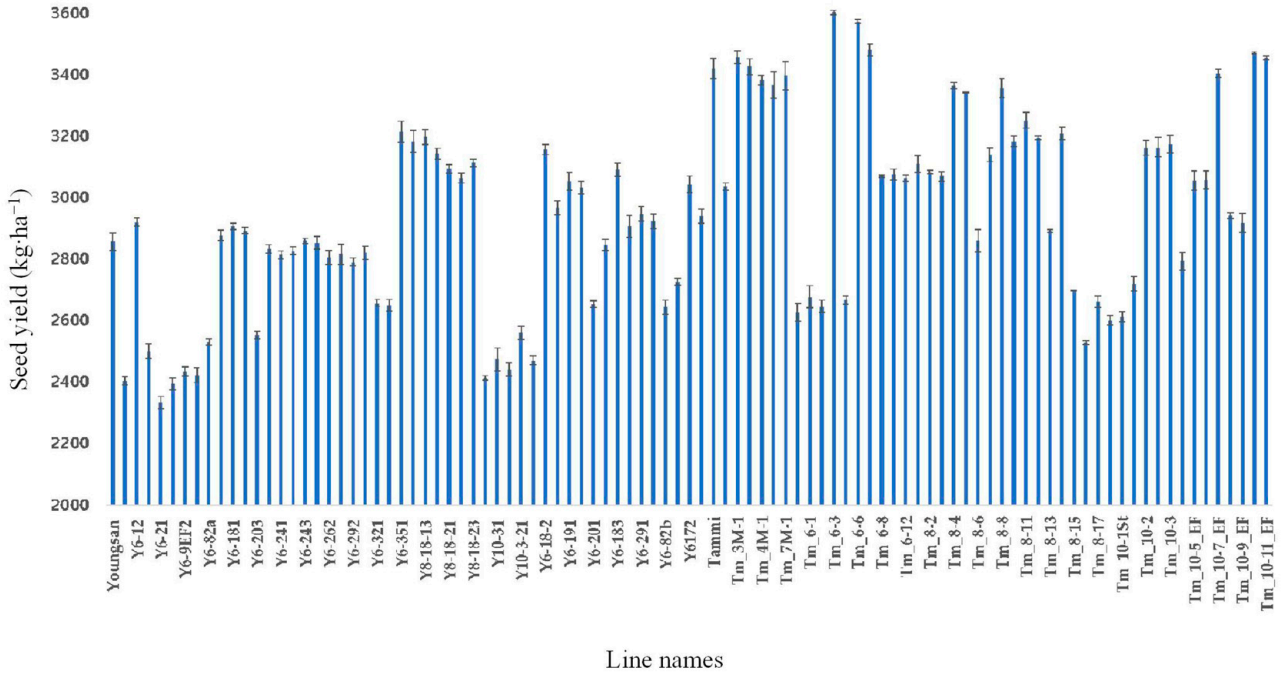

Figure 4. Seed yield in the two original cultivars and 95 mutant lines.

The total phenolic contents (TPCs) of the 95 mutant lines and the two original cultivars are shown in Figure 5. The TPCs of 'Tammi' and 'Youngsan' were 57.29 ± 0.78 mg·g^{-1} and 66.04 mg·g^{-1}, respectively. The highest TPC was in Y6-81a (78.31 mg·g^{-1}), and the lowest TPC was in Y6-29-30 (15.86 mg·g^{-1}). Fourteen mutant lines had lower TPC (<30 mg·g^{-1}): Y8-18-23, Y10-3-21, Y6-81b, Y6-201, Y6-202, Y6-291, Y6172, Y6-29-30, Tm8-14, Tm10-1, Tm10-2, TM10-3, Tm10-4EF, and Tm10-5EF. Two mutant lines, Y6-11 and Y6-81a, showed significantly higher TPCs than that of their original cultivar 'Youngsan'.

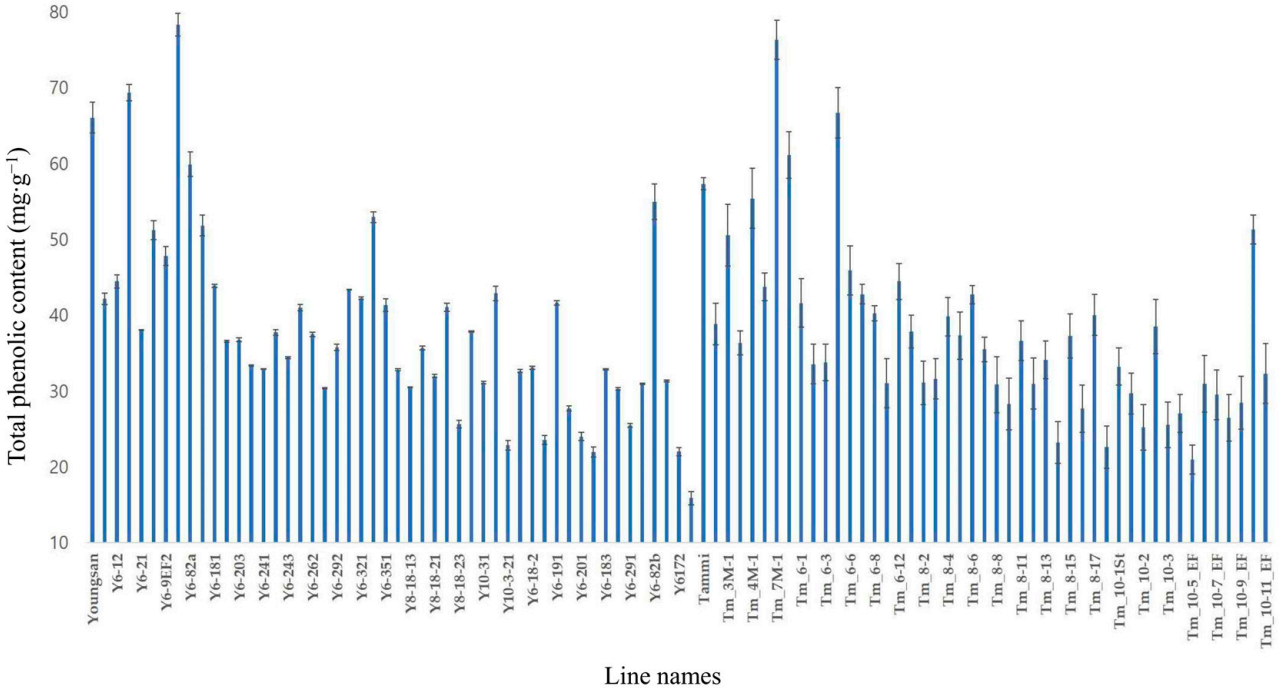

Figure 5. Total phenolic compound contents in 97 rapeseed genotypes (n = 3 per genotype).

3.4. Association Analysis

All of the SNPs significantly associated with phenolic compounds and glucosinolate (progoitrin) are listed in Table S6. Among the 70,208 union SNP dataset associations identified, 241 SNPs were significantly associated with phenolic compounds and glucosinolate. Of the 241 selected union SNPs, 119 were located in genic regions, and 122 were located in intergenic regions. Two candidate genes associated with the strongest SNP variant for two major phenolic compounds (*trans*-sinapine 1 and *trans*-sinapine 2) were identified. *BnaCnng39930D* encoding a RING/U-box superfamily protein was associated with *trans*-sinapine 1, and *BnaA07g31720D* encoding a pentatricopeptide repeat (PPR) superfamily protein was associated with *trans*-sinapine 2. Five SNP loci were significantly associated with total sinapine content (TSC). The genes at the five loci were *BnaAnng09880D* (no annotated function), *BnaC06g38030D* (transcription coactivator), *BnaC03g31950D* (S-adenosyl-L-methionine-dependent methyltransferase superfamily protein), *BnaC05g08990D* (RNA-binding; RRM/RBD/RNP motifs family protein), and *BnaA06g09180D* (protein kinase 2A). The C03_19651313 SNP marker on chromosome C03 was consistently associated with both TSC and sinapoyl malate, and the gene at this locus was *BnaC03g31950D*. The candidate gene most strongly associated with variation in progoitrin content was *BnaC08g30570D* (ARABIDILLO-2) (Tables 3 and 4). No SNPs were significantly associated with variations in seed yield.

Table 3. A summary of the SNPs significantly associated with phenolic compounds.

Compounds	SNP No.	SNP Loci
Dihexose	5	*BnaC02g20420D*, *BnaA03g40510D*, *BnaA03g18260D*, *BnaC03g32440D*, *BnaC03g20990D*
Progoitrin	15	*BnaC08g30570D*, *BnaC06g02290D*, *BnaC06g02290D*, *BnaA06g31740D*, *BnaC01g10570D*, *BnaA01g13400D*, *BnaC04g51220D*, *BnaA08g28090D*, *BnaC07g03420D*, *BnaC01g13560D*, *BnaC01g04810D*, *BnaC05g24360D*, *BnaAnng10510D*, *BnaA08g24650D*, *BnaC09g27750D*
trans-sinapine 1	27	*BnaCnng39930D*, *BnaC02g35370D*, *BnaC06g07520D*, *BnaA09g13790D*, *BnaAnng26190D*, *BnaC05g11590D*, *BnaC08g35930D*, *BnaA09g19450D*, *BnaA06g07300D*, *BnaA06g08830D*, *BnaCnng18490D*, *BnaC06g38030D*, *BnaA08g09430D*, *BnaC06g08200D*, *BnaCnng23620D*, *BnaA10g22100D*, *BnaCnng58110D*, *BnaA01g20360D*, *BnaA07g37760D*, *BnaC04g10570D*, *BnaA09g24030D*, *BnaA06g09240D*, *BnaC04g10570D*, *BnaA08g25950D*, *BnaC05g46690D*, *BnaA07g31720D*, *BnaC03g31950D*
trans-sinapine 2	1	*BnaA07g31720D*
Total sinapine content	5	*BnaC06g38030D*, *BnaC05g08990D*, *BnaC03g31950D*, *BnaAnng09880D*, *BnaA06g09180D*
Sinapine(4-*O*-8′)guaiacyl	13	*BnaA09g16810D*, *BnaA05g23210D*, *BnaC07g23750D*, *BnaCnng31150D*, *BnaC09g23720D*, *BnaC09g22600D*, *BnaC08g16340D*, *BnaC03g49370D*, *BnaC01g17020D*, *BnaA08g06400D*, *BnaC02g26880D*, *BnaC09g54330D*, *BnaC07g23750D*
Methyl sinapate	41	*BnaCnng46600D*, *BnaCnng42240D*, *BnaCnng31900D*, *BnaCnng20310D*, *BnaCnng13750D*, *BnaC09g41550D*, *BnaC09g26810D*, *BnaC09g24670D*, *BnaC09g17390D*, *BnaC07g04160D*, *BnaC06g18890D*, *BnaC06g18590D*, *BnaC06g07440D*, *BnaC06g07070D*, *BnaC06g06790D*, *BnaC05g04580D*, *BnaC04g56040D*, *BnaC03g70630D*, *BnaC03g50270D*, *BnaC03g32630D*, *BnaC03g09080D*, *BnaC02g16010D*, *BnaC01g41960D*, *BnaC01g21850D*, *BnaC01g16590D*, *BnaC01g05320D*, *BnaAnng07310D*, *BnaA10g14470D*, *BnaA10g11420D*, *BnaA10g00800D*, *BnaA09g16810D*, *BnaA08g12900D*, *BnaA04g14130D*, *BnaA03g31990D*, *BnaA01g27460D*, *BnaA01g25280D*, *BnaA01g19380D*, *BnaA01g19380D*, *BnaA01g19380D*, *BnaA01g19380D*, *BnaA01g06890D*
Sinapoyl malate	1	*BnaC03g31950D*
Total phenolic content	2	*BnaC01g06630D*, *BnaAnng27700D*

Table 4. Association study highlighting candidate genes associated with phenolic compounds in rapeseed mutant lines.

Triats	Chr_Position	LOG10(P)	Transcript:Feature	Description	TAIR ID	Allele
DI [a]	C02_16859648	4.70	*BnaC02g20420D*:CDS	Ulp1 protease family protein	AT5G45570	T/G
MS [b]	A01_ 19200278	6.74	*BnaA01g06890D*:CDS	Heavy metal atpase 2	AT4G30110	C/T
Pro [c]	C08_30292042	5.66	*BnaC08g30570D*:CDS	ARABIDILLO-2	AT3G60350	A/T
Pro	A06_21272025	4.68	*BnaA06g31740D*:CDS	Tetratricopeptide repeat (TPR)-like superfamily protein	AT3G27960	C/T
SG [d]	A09_10109576	6.52	*BnaA09g16810D*:Intron	GroES-like zinc-binding dehydrogenase family protein	AT5G43940	T/C
S1 [e]	CNN_38500012	6.42	*BnaCnng39930D*:CDS	RING/U-box superfamily protein	AT2G15530	G/A
S2 [f]	A07_22086512	4.59	*BnaA07g31720D*:CDS	Pentatricopeptide repeat (PPR) superfamily protein	AT1G79590	G/T
TSC [g]	ANN_10591701	4.47	*BnaAnng09880D*:CDS	No annotated function	-	A/T
TSC	SC06_35793928	4.33	*BnaC06g38030D*:CDS	Transcription coactivators	AT1G77320	G/T
TSC	C03_19651313	4.25	*BnaC03g31950D*:Intron	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	AT4G00740	T/G
SM [h]		4.59				
TSC	C05_4843154	4.21	*BnaC05g08990D*:promoter	RNA-binding (RRM/RBD/RNP motifs) family protein		G/A
TSC	A06_4969999	4.07	*BnaA06g09180D:Intron*	Protein kinase 2A		A/T
TPC [i]	ANN_31705069	5.39	BnaAnng27700D:promoter	Syntaxin of plants 52	AT1G79590	G/T

[a] DI: dihexose, [b] MS: methyl sinapate, [c] Pro: progoitrin, [d] SG: sinapine(4-*O*-8′)guaiacyl, [e] S1: *trans*-sinapine 1, [f] S2: *trans*-sinapine 2, [g] TSC: total sinapine content, [h] SM: sinapoyl malate, [i] TPC: total phenolic content.

3.5. Networking Analysis

The relationships among the genes identified in the association analysis were analyzed using GeneMANIA (Figure 6). Among the four types of functional interaction networks, co-expression accounted for the largest proportion of identified genes (96.05%), followed by co-localization (2.14%), predicted interactions (1.67%), and shared protein domains (0.14%). Co-expression was predicted to affect the TPC in rapeseed. The GeneMANIA analyses did not detect any direct network linking defensin-like (DEFL) family protein (AT2G04045; *BnaC02g35370D*), double Clp-N motif-containing P-loop nucleoside triphosphate hydrolases superfamily protein (AT4G29920; *BnaAnng26190D*), AT59 (*BnaA06g09240D*), or MEI1 (*BnaC06g38030D*). Twenty associating genes were identified, which are listed according to rank order as follows: CFM3A (No. 1), PDE312 (No. 2), PUB17 (No. 3), ATEXO70E1 (No. 4), FTSH12 (No. 5), CLASP (No. 6), AT3G59040 (No. 7), AT2G32170 (No. 8), emb1703 (No. 9), SKIP8 (No. 10), CITRX (No. 11), RH39 (No. 12), LECRK55 (No. 13), EMB2654 (No. 14), AT5G58330 (No. 15), GTE2 (No. 16), RIBA1 (No. 17), ACT8 (No. 18), MEE40 (No. 19), and PUB13 (No. 20). Interestingly, TPR13 (tetratricopeptide repeat-like superfamily protein) was consistently associated with sinapine 1, sinapine(4-*O*-8′)guaiacyl, and progoitrin, while AT4G00740 (probable methyltransferase PMT13) was significantly associated with TSC and sinapoyl malate.

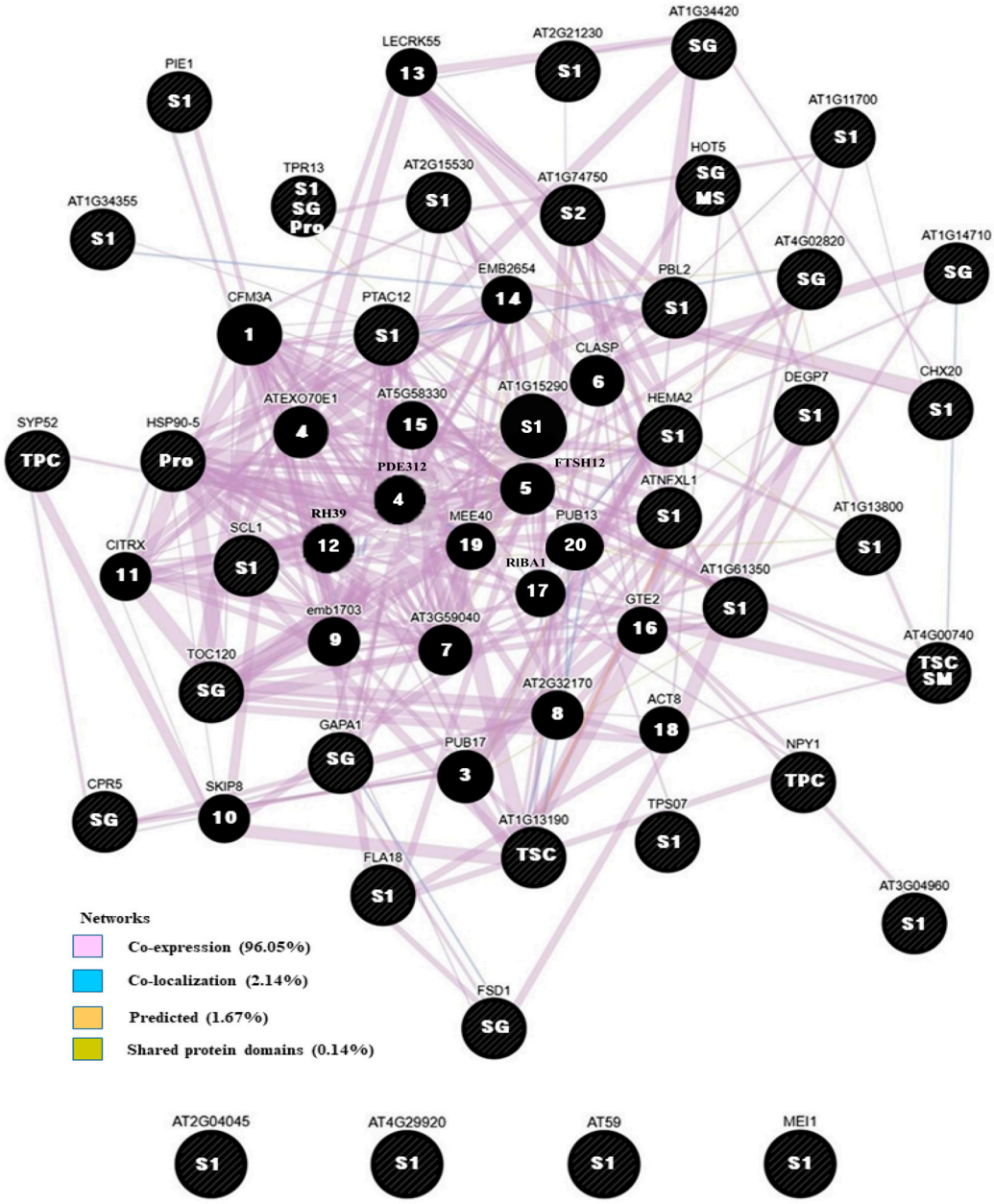

Figure 6. Networking analysis of phenolics and glucosinolate-associated genes harboring SNPs, constructed using GeneMANIA. S1: *trans*-sinapine 1, S2: *trans*-sinapine 2, SG: sinapine(4-*O*-8′)guaiacyl, SM: sinapoyl malate, TPC: total phenolic content, TSC: total sinapine content, MS: methyl sinapate, Pro: progoitrin, 1–20: rank number.

4. Discussion

Rapeseed is a widely cultivated oil crop, and its seeds are also a valuable food, feed, and source of materials for the bio-industry [1,2]. Phenolic compounds and glucosinolates are widely distributed in rapeseed. Their antioxidant properties have been demonstrated using a range of assay methods, including 2, 2′-diphenyl-1-picrylhydrazyl radical (DPPH), ferric reducing antioxidant power, peroxyl radical scavenging, ferric tripyridyl triazine complex reduction, and organic radical scavenging assays [1–7]. They are associated with a number of health benefits, including reduced risks of chronic diseases such as cancer, cardiovascular disease, and diabetes [6,7]. However, phenolic compounds and glucosinolates in rapeseed confer a bitter taste and decrease palatability, thereby making food and feed less desirable [5,6]. Despite the nutritional and medicinal value of phenolic compounds and glucosinolates, there is little information available about rapeseed genetic

resources or genetic markers associated with these traits. This knowledge gap restricts the manipulation of these traits in breeding programs.

Generally, the variations in phenolic compounds in rapeseed mainly depend on genotype, while small variations have been attributed to cultivation conditions [29,30]. Typically, new genetic resources for rapeseed are developed through hybridization. However, the narrow range of phenolic compounds in rapeseed genotypes has limited the accurate characterization of new genetic resources [6,7,29,30]. Mutagenesis using gamma-ray radiation is an effective way to increase the variation in phenolic compounds in various plants. Gamma-ray mutagenesis has been used to improve many useful traits of rapeseed, such as seed yield, morphology, resistance to pathogens, and variability in fatty acid composition [14,15]. Our research group recently developed new cultivars of *Chrysanthemum*, *Rubus*, and *Dendrobium* with altered phenolic compound profiles using gamma-ray irradiation [16,31,32]. In this study, we analyzed the phenolic compound profiles of two original cultivars and 95 mutant lines of rapeseed obtained using gamma-ray irradiation. The original cultivars 'Youngsan' and 'Tammi' are mid-oleic acid (63.0–66.0%), low glucosinolate (0.43%), mid-oil content (43.9%), and non-erucic acid [33] cultivars. Several studies have investigated the oil content, fatty acid composition, and glucosinolates in rapeseed; however, few have focused on phenolic compounds [5,34,35]. The major phenolic compounds in the original cultivars and mutant lines are sinapine [33]. The major phenolic compounds in the original cultivars and mutant lines are sinapate esters with sinapoylcholine (sinapine) [33]. Sinapine is the most abundant phenolic compound in rapeseed, accounting for more than 65% of the TPC [29,33–36]. There have been no previous studies on the phenolic compound contents in Korean breeding cultivars and mutant genotypes of rapeseed. The sinapine concentrations in the original cultivars 'Youngsan' and 'Tammi' (29.86 and 33.68 $mg \cdot g^{-1}$, respectively) were higher than those reported for 575 winter rapeseed genotypes (3.2 to 12.7 $mg \cdot g^{-1}$) [37], but similar to or lower than those reported for two Korean domestic cultivars and one imported cultivar from India (29.74 to 52.24 $mg \cdot g^{-1}$) [36]. Reducing the sinapine content has been one of the major goals in rapeseed breeding to improve the nutritional value of rapeseed meal. The high TPCs in the original cultivars 'Youngsan' and 'Tammi' mean that their seeds have low value in the food and feed industries. The TPCs in eight mutant lines were less than half of those in the original cultivars, whereas the TPC in Y6-81a was significantly higher than those in the other genotypes. A previous study reported that sinapine was the chief contributor to antioxidant activity in Australian canola meal [7]. The ability to control the TPC with radiation breeding may have applications in rapeseed breeding programs for the food, feed, and medicinal industries. Additionally, phenolic compounds determine the seed coat color of rapeseed [6]. In the present study, the original cultivars had a black seed coat. Although the mutants had increased or decreased phenolic compound contents compared with the original cultivars, only one showed a different seed coat color (Y6-29-30, which produced brown seeds). Mutation breeding technology can change some characteristics of a pre-existing cultivar and improve its nutritional quality [10]. High seed yield is an important trait for rapeseed breeding [37]. The original cultivars 'Youngsan' and 'Tammi' have favorable agronomic characteristics, such as early flowering and mid-seed yield (2800 to 3500 kg/ha). Evaluation of the mutant lines over three growing seasons showed that the seed yield of two mutant lines (Y6-351 and Y8-18-13) was 10% higher (>3200 kg/ha) than that of their original cultivar 'Youngsan'. Unfortunately, none of the SNPs detected in the GBS analysis were significantly associated with seed yield. Nevertheless, the substantial improvement in seed yield observed in Y6-351 and Y8-18-13 suggests their potential utility in the development of new cultivars.

NGS methods have been used in molecular research on rapeseed [13,21]. However, information about changes in the types and contents of phenolic compounds in rapeseed genotypes produced using mutation breeding is lacking. In this study, we explored novel SNPs in rapeseed mutant lines generated using gamma-ray irradiation. The number of SNPs ranged from 417 to 11,256, averaging 6649 per mutant line. In the rapeseed mutant lines, many SNPs were detected on chromosomes C01, C02, and C03. Similarly, in our pre-

vious study, many SNPs were detected on chromosomes C01, C03, and A09 in mutant lines with altered fatty acid profiles generated with gamma-ray irradiation [21]. The biological effect of gamma rays depends on the genotype, and chromosome aberration is the most important event in mutation breeding [14]. Of the 70,208 union SNPs detected in this study, 241 were significantly associated with seven phenolic compounds. Association studies based on SNP markers have been widely used to analyze complex quality traits in crops. Previous studies have identified candidate genes associated with oil content, fatty acids, glucosinolate, sinapine, phytic acid, tannin, and crude fiber in rapeseed [13,15,21,37,38]. The present study identified significant SNPs associated with phenolics (sinapine, sinapoyl malate, and methyl sinapate) and glucosinolates (progoitrin) in various mutant lines using association analysis. Then, the networks involved in the genetic control of these traits were explored using GeneMANIA. Phenolics are responsible for the bitter taste of vegetables, and they can induce hypothyroidism. However, these compounds are of interest because of their potential as cancer-preventive agents [39]. The glucosinolate content in rapeseed appears to be influenced by genetic and environmental factors, as well as by nitrogen supply. Nitrogen fertilizer is required for growing rapeseed with low glucosinolate contents, but this can be expensive [29,40]. The development of novel rapeseed cultivars with low glucosinolate content can solve this problem. In this study, a total of 21 genes were associated with progoitrin, with *BnaC08g30570D* (ARABIDILLO-2; ARD-2) identified as a strong candidate gene. However, further research is needed to fully understand the role of *BnaC08g30570D* in glucosinolate biosynthesis. The network analysis also identified a tetratricopeptide repeat (TPR)-like superfamily protein associated with glucosinolate biosynthesis. TPR-like superfamily proteins have no known direct connections with glucosinolates. However, the TPR domain is often found in conjunction with the F-box domain or WD40 domain, and proteins with these domains are involved in DNA repair [41,42]. Therefore, it is possible that a TPR protein could also play a role in regulating glucosinolate biosynthesis in plants.

Sinapine biosynthesis is a complex biochemical pathway in plants that leads to the production of sinapine, also known as sinapoylcholine [4,5,7]. The gene *BnaCnng39930D* containing an SNP associated with *trans*-sinapine 1 was predicted to encode a RING/U-box superfamily protein. The RING/U-box superfamily is a large and diverse group of E3 ubiquitin ligases that play important roles in regulating various cellular processes, including protein degradation, transcriptional regulation, and signal transduction [43]. While there is no direct evidence that RING/U-box proteins are related to phenolic biosynthesis in plants, *BnaCnng39930D* is known to be involved in the biosynthesis of erucic acid, one of the fatty acids in the seed oil of rapeseed [43]. Interestingly, our previous study revealed that oleic acid is the main fatty acid in most mutants derived from 'Tammi', except for Tm8-15 and Tm10-1St. Oleic linoleic and erucic acid were identified as the major fatty acids in Tm8-15 and Tm10-1St [21]. In the present study, these mutants also showed significantly lower sinapine contents. Further research is needed to fully understand the role of RING/U-box proteins in phenolic biosynthesis. The gene *BnaA07g31720D* containing the *trans*-sinapine 2-related SNP was predicted to encode a PPR superfamily protein. Members of the PPR superfamily are RNA-binding proteins that are involved in various aspects of RNA metabolism, including RNA editing, splicing, and translation. A study on *Arabidopsis* suggested that PPR proteins may indirectly affect the biosynthesis of sinapine by regulating the expression of genes involved in its biosynthetic pathway [44]. A PPR protein has been shown to regulate the expression of genes involved in the phenylpropanoid pathway, including the gene encoding cinnamate 4-hydroxylase (C4H), which is also a key enzyme in sinapine biosynthesis. Similarly, the PPR protein OTP70 has been shown to regulate the expression of genes involved in the phenylpropanoid pathway in rice [45]. Thus, the PPR superfamily protein encoded by *BnaA07g31720D*, which affects sinapine content, may function in a similar manner. *BnaA01g06890D*, which contains a methyl sinapate-related SNP, was predicted to encode heavy metal ATPase 2 (HMA2). HMA2 plays a role in heavy metal detoxification in plants by transporting heavy metal ions such as copper, zinc, cad-

mium, and lead out of the cytosol and into intracellular compartments or out of the cell [46]. Compared with wild-type plants, HMA2-knockout mutants of *Arabidopsis thaliana* exhibited reduced levels of total phenolic compounds, including flavonoids and lignin [47]. However, the exact mechanism by which HMA2 influences phenolic biosynthesis in rapeseed is not yet known.

The association analysis revealed that *BnaC06g38030D* (transcription coactivator) *BnaC03g31950D* (S-adenosyl-L-methionine-dependent methyltransferase superfamily protein), *BnaC05g08990D* (RNA-binding; RRM/RBD/RNP motifs family protein), and *BnaA06g09180D* (protein kinase 2A) showed significant associations with TSC. The biosynthesis of sinapine is mediated by a number of enzymes, including protein kinases. Protein kinases catalyze the transfer of a phosphate group from ATP to a target protein, typically another enzyme or a receptor protein [48]. In the context of sinapine biosynthesis, protein kinases are involved in the regulation of the pathway, where they control the activity of the participating enzymes. A serine/threonine kinase has been identified to play a role in sinapine biosynthesis in rapeseed [4,5,48]. Protein kinases regulate diverse cellular processes such as signal transduction, cell division, and metabolism. There is evidence to suggest that phenolic compounds interact with protein kinases and modulate their activity [49,50]. Phenolic compounds inhibit the activity of some protein kinases but promote the activity of others. In addition, certain phenolic compounds can alter the expression of genes encoding protein kinases, potentially leading to changes in cellular signaling [48,50]. The biosynthesis of sinapine involves several steps, one of which is catalyzed by S-adenosyl-L-methionine (SAM)-dependent methyltransferases (SAM-Mtases). SAM-Mtases transfer a methyl group from SAM to a target molecule, usually a protein, DNA, RNA, or small molecule [49–52], and play a crucial role in many biological processes, including gene expression, signal transduction, and metabolism [22,52]. In sinapine biosynthesis, SAM serves as a methyl donor from which SAM-Mtases transfer the methyl group to a precursor molecule, sinapoylglucose, forming sinapoylmalate. This reaction is catalyzed by malate sinapoyltransferase (SMT), which belongs to the SAM-Mtase superfamily [5,22,52]. SMT is a key enzyme in the biosynthesis of sinapine and is expressed in several plant tissues, including leaves, stems, and seeds [51]. Its activity is regulated by various factors, including developmental stage, environmental conditions, and genetic background. SAM-Mtases play essential role in the biosynthesis of sinapine and other phenolic compounds in *Brassica* species [50–52]. However, the candidate genes encoding these enzymes are members of multiple-gene families. A clear understanding of the relationships among them is necessary to elucidate the functional mechanism of phenolic compound biosynthesis in rapeseed.

In this study, as part of the genome analysis, we used GBS analysis to identify associations between mutations in rapeseed and phenolic compounds and to investigate candidate genes linked to these associations. Although radiation-induced mutations are known to occur independently at different genetic loci, our previous research revealed the presence of mutation hotspots where mutations occur in the same genomic regions [53]. To enhance the precision of our genetic analysis, it is imperative to conduct quantitative trait loci (QTL) analysis to investigate linkage disequilibrium within the mapping population. Additionally, we propose the integration of advanced NGS technologies, such as whole-genome sequencing and targeted resequencing, with GBS analysis to gain a more comprehensive understanding of these genetic phenomena.

5. Conclusions

In the present study, we analyzed phenolics and glucosinolates (progoitrin) in the seeds of novel rapeseed mutants. There were differences in phenolic and progoitrin profiles between the two original cultivars ('Youngsan' and 'Tammi') and among their gamma-irradiated mutants. Changes in the types and amounts of these compounds are the focus of breeding programs to improve the quality of rapeseed. The results of our study identified mutant genotypes and 241 candidate genes significantly associated with phenolic quality traits of rapeseed. This information may offer helpful insights to promote rapeseed

breeding. This is the first study describing the SNPs generated using GBS and an association analysis to identify genes related to the large variability in phenolic contents among rapeseed mutants derived from gamma-ray irradiation. *BnaCnng39930D*, *BnaA07g31720D*, *BnaC03g31950D*, *BnaA06g09180D*, and *BnaA06g31740D* were identified as candidate genes related to sinapine and progoitrin contents. Studies on the molecular networks that regulate glucosinolates and phenolic compound metabolism in mutants obtained using radiation breeding techniques can provide valuable insights into the regulation of plant secondary metabolism. The mutants obtained in this study will be useful resources for the development of novel rapeseed cultivars with improved phenolic and glucosinolate profiles. Our results may help researchers select mutants with suitable SNPs, associations, and networks as optimal genotypes for the food and medicinal industries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9111204/s1, Table S1: Results of genotyping-by-sequencing analysis in rapeseed genotypes. Table S2: Summary of total SNP numbers and alignment to the reference genome sequence. Table S3: Summary of polymorphic SNPs collected by comparing the common SNPs in the original cultivar with the base sequences of the mutant lines. Table S4: List of union SNP matrix loci that were generated for 95 rapeseed mutant lines. Table S5: Peak assignments of the aqueous methanol extract from the seed of rapeseed. References [7,36] are cited in the Supplementary Materials. Table S6: List of 241 significant associated SNPs with phenolics by association mapping in rapeseed. Supplement Material File S1: List of polymorphic SNPs in 95 mutant lines associated with phenolic compounds by association study in rapeseed mutant line.

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